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INFORMATION REPORT INFORMATION REPORT

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SUBJECT Research on Protein Compounds of Vitamin B_{12}

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1. a report on a Polish research project (11 pages, English), entitled "Protein Compounds of Vitamin B_{12} and its Analogs" which is being conducted at the College of Agriculture located at Poznan. Dr J Janicki is in charge of the project.

2. The object of this research is to prepare by biological procedures and to isolate protein or peptide complexes of vitamin B_{12} and its analogs from bacterial cultures and other sources; identify the analogs and the amino acids present in the complex and to test the biological content of the preparations. The plan of work calls for procedures to be developed for the isolation of protein complexes of vitamin B_{12} from cultures of different species of Propionic acid bacteria, rumen contents and city sludges. The characteristics of the protein portion of the complex and the cobalamin residue in the analogs of the vitamin, and the light sensitive forms are to be determined. Biological activity of the isolated complexes and analogs will be tested by injecting them into eggs low or devoid of vitamin B_{12} and checking the effect on embryo development. FOR OFFICIAL USE ONLY

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PROTEIN COMPOUNDS OF VITAMIN B_{12} AND ITS ANALOGS

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Foreign grant No.: FG - Po - 137 - 62 50X1-HUM
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S u m m a r y

Althoug the structure of vitamin B_{12} is now well established, little is known about the mechanism of biological action of different corrinoids. In connection with the recently reported data on the isolation of various cobamide coenzymes, as well as of a number of protein bound corrinoids, an extensive research was undertaken, which may throw more light on the question, whether all the native forms of these compounds are bound with different protein fractions, or they are formed artificially depending on the mode of isolation.

Propionibacterium shermanii 1 were used as source of these complexes. The ability of these bacteria to produce corrinoids in relactively large amounts would be a good explanation of the choice of experimental material. P.shermanii was grown in two different media, based on the acid and enzymic hydrolyzates of casein and on the yeast autolysates. The preliminary attempts to isolate the protein compounds of vitamin B_{12} from the bacterial cells failed. The adopted isolation procedure, eryginaly applied to animal material, appeared to be unsuitable. Further research included the examination of a method which has been used in research on the protein bound vitamin B_{12} contents in food supplement of bacterial origine. This method has been slightly modified. Both the original and modified purification procedures led to isolation of an amorphus material which was then subjected to further studies. The preliminary characteristics of the products included: the measurements of the absorption spectra in the range of 200-700 mμ, the electrepheretic

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check on the homogenity and mobility, the amino acid composition prior and after acid hydrolysis. In both isolated compound the presence of a protein fraction has been evidenced. To obtain reproducible and interpretable data further studies are required.

Introduction:

The recent years brought a rapid progress in the biochemistry of corrinoids and related compounds. The investigations devoted to the occurance, nature and structure of these compounds could be classified into one of the following groups:

a/ dealing with the isolation of corrinoid-protein complexes from various natural sources, without further st studies on their enzymic character and activity,

b/ concerning the structure and functions of different cobamide coenzymes and holoenzymes, showing specific activities in some enzymic reactions.

Wijmenga /1/, Hedbom /2/ and Hausmann /3/ succeeded with isolation from animal liver of a corrinoid fraction containing protein. Different complexes of this type were also found in pig whey /4/, bovine blood /5/, food supplement /6/ and in rabbit feces. The isolation and purification of a vitamin B_{12} protein complexes /erythroglobulin/ from bovine blood serum was also reported /8/. These findings could be rather considered as being an approach to the problem of the "Intrinsic Factor" /9/. Pertaining the class of compounds mentioned under b/ it has to be noted that in most cases the catalitic activity of the isolated B_{12}-coenzymes is now well defined /10/. However relatevely little is known about the "apoenzymic" parts of the various holoenzymes /B_{12}-enzymes/. All the light sensitive and coenzymic forms of corrinoids may occur

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in nature as bound with different proteins or polypeptides. This hypothesis was recently supported by establishing the existence of protein-bound corrinoids compounds capable to catalyse the synthesis of methionine /11/. Generally, the following aspects of the corrinoids chemistry should be taken into consideration in carring out research on their native forms successfully:

1. the ability of forming artefacts, due to the isolation procedure /the stability toward different conditions including pH, temperature and effect of light/,
2. the relationship between the valency of cobalt atom and the biological activity of the corrinoids,
3. the possible modes and sites of binding the protein portion.

Experimental:

Materials and methods

Bacteria - Propionibacterium shermanii 1 was used as source of the corrinoid-protein compounds. This strain is being stored as stick culture in a yeast-glucose medium supplemented with casein hydrolyzate /12/. Cultures were incubated using 5 l liquid media.

Two different composition of the medium were applied:

Medium No 1:

1. acid hydrolyzate of casein /1.980 g N/100 ml/.... 100 ml x/
2. enzymic hydrolyzate of casein /0.996 g N/100 ml/. 200 ml x/
3. K_3PO_4 /4.4 g in 100 ml of H_2O/..... 20 ml
4. NaH_2PO_4 /4.4 g in 100 ml of H_2O/..... 20 ml

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5. $MgCl_2 \cdot 6H_2O$ /2 g in 50 ml of H_2O/..... 10 ml
6. $CoSO_4 \cdot 7H_2O$ /0.1 g in 250 ml of H_2O/..... 30 ml
7. destilled water..... 560 ml

After adjusting the pH to 6.5, the solution was sterilized for 30 min. at 120°C.

The following components were subsequently added in sterile conditions:

8. $FeSO_4 \cdot 7H_2O$/100 mg in 100 ml of H_2O/..... 10 ml
9. calcium pantothenate /100 mg in 250 ml of H_2O/... 10 ml
10. biotin /15 mg in 500 ml of H_2O/..... 10 ml
11. glucose /60% solution/..... 30 ml

x/ The acid and enzymic hydrolysates of casein were prepared in this laboratory using concentrated HCl and trypsin, respectively.

Medium No 2:

1. Yeast autolysate..... 350 ml[xx]
2. Malt extract /~~beverage~~/, 12°Bg..... 75 ml
3. K_3PO_4 /64 g in 240 ml of H_2O..... 40 ml
4. KH_2PO_3 /36 g in 360 ml of H_2O..... 30 ml
5. NaH_2PO_4 /64 g in 240 ml of H_2O/..... 30 ml
6. $MgCl_2 \cdot 6H_2O$ /10 g in 250 ml of H_2O/..... 10 ml
7. $ZnSO_4$ /80 mg in 200 ml of H_2O/..... 10 ml
8. $CoSO_4 \cdot 7H_2O$ /100 mg in 250 ml of H_2O/..... 30 ml
9. $CuSO_4$ /200 mg in 400 ml of H_2O/..... 10 ml
10. distilled water..... 375 ml

The pH was brought to 6.5 and the solution was sterilized for 15 min. at 110°C.

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Subsequently the following components were added in sterile conditions:

11. $FeSO_4 \cdot 7H_2O$/100 mg in 100 ml of H_2O/..... 100 ml

12. glucose /60% solution/..... 30 ml

xx/ The autolysate from brewer yeast /1 l from 1 kg/ as well as the malt extract has been prepared in this laboratory.

To each of the 4 l culture media 20% /1000 ml/ of inoculum was added. This inoculum has been previously three times transplanted /in two day intervals/. The bacteria were cultivated at 30°C for 8 days in the case of "casein" medium and 5 days in that of yeast autolysate. After each of 24 hrs of incubation the dropping pH has been adjusted to 6.8 - 7.0 by means of concentrate ammonia and 20 ml of 60% glucose solution were added per 1 l of the medium. 16 mgs of 5,6-dimethylbenzimidazole per 1 l of the culture were also added after 48 hrs of incubation. The cells were harvested by centrifugation, washed with distilled water and recentrifuged. 1 l culture yielded ca 47.5 g of fresh cell paste. The material was stored in low temperatures /-20°C/.

Isolation and purification of protein containing corrinoids:

Procedure 1: /according to Mulli and Schmidt /6//

Extraction was performed by means of 75% ethyl alcohol at room temperature, using a Warring blendor. The suspension was centrifuged and the residual material subjected to further similar extractions. The combined supernatants were evaporated at 30°C under reduced pressure in order to remove the ethanol. To the water solution, after bringing it to a given volume, 275 g of solid ammonium sulphate were added per 1 l. When a clear solution was obtained, several extractions were carried out with

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small portions of n-butanol in order to isolate quantitatively the colored compounds. The butanol extracts were combined and clarified by centrifugation. The supernatant was passed through a column of Al_2O_3 /commercial product, Gliwice, Poland/ previously activated by means of acetic acid /pH 6/ and dried at 130°C for 1 hour. The column was eluted first with 1000 ml of 90% acetone and then with 1000 ml of 80% acetone. This operation was followed by passing distilled water, resulting in elution of a fraction showing intensive red color. The aqueous solution was evaporated under reduced pressure to remove residual acetone. Each 100 ml portion of the solution has been treated with 37 g of ammonium sulphate and subjected to additional extraction with n-butanol. The n-butanol extract was rechromatographed and the red agueous eluate shaked with small volumes of phenol. The combined phenol extracts were carefully washed with distilled water, then treated with nine volumes of acetone and allowed to stand for several hours. An amorphous red precipitate was seperated by centrifugation, washed with alcohol an ether, and dried over H_2SO_4 in a dissicator. This material was subjected to further studies.

Procedure 2:

By adopting the Mulli and Schmidt conditions to isolate the protein containing corrinoids, observations were made concer - ning the uncomplete butanol extraction of the colored compounds. The aqueous solution treated with ammonium sulphate, after several extractions with n-butanol, still contained a lot of a colored material. Attemps were made in order to recover quantitatively this material. The aqueous solution treated with ammonium sulphate, after complete extraction with n-butanol, was

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distilled water. Subsequently a phenol extraction was performed. After washing with water to the combined phenol extracts, an equal volume of ethyl ether was added. The colored compounds were extracted by means of small portions of destilled water. In order to remove the remaining amount of phenol, the water solution thus obtained, has been carefully washed with ethyl ether. The solution was then evaporated under reduced pressure to get free of the ether, and passed through a column of Al_2O_3 as described under "Procedure 1". All the impurities showing brown color remained on the column, whilest the red "passed through" solution was again extracted with small portions of phenol. The phenol layer was washed with distilled water and treated with 9 volumes of acetone. The solution was left at $4^{\circ}C$ for several hours, during which time it yieldded an amorphus material, red in color. After centrifuging it and washing with alcohol and ethyl ether, the precipitate was dried and stored in a desiccator over H_2SO_4. Other methods applied in this research are described or referred to in appropriate section of the text.

Result and discussion

Each of the procedures, described in the "Experimental", yielded a few of milligrammes of an amorphus material. The following preliminary identification tests were performed:

1. Qualitative reaction on protein:

The specific protein test with Folin and Ciocalteu reagent according to Lowry et al./13/ gave negative result. A slightly blue color was observed when cristalline vitamin B_{12} was treated with this reagent.

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2. The amino acid composition after hydrolysis:

The material was hydrolyzed by means of 6 N HCl /analytical grade/ in a tube sealed in nitrogen atmosphere. A ratio 500:1 /by weight/ of the acid to the material was used. Hydrolysis was carried out at 110°C for 22 hrs. At the end of hydrolysis the hydrochloric acid has been evaporated under reduced pressure. After addition of distilled water /1/4 volume of the acid used/ the solution was evaporated again and the residue treated with pH 2.2 citrate buffer 0.2 N. The undissolved material was removed by filtration whilest the clear filtrate was analyzed chromatographically. A load coressponding to 2 mg of the material was applied. The amino acid composition of the hydrolyzates thus obtained has been determined using an automatic recording apparatus /Phoenix Precision Inst.,Co.,Model K-5000-A/. The operation of this instruments is based on the method of Moore, Spackman and Stein /14/. The neutral and acidic amino acids are separated on a 150-cm.column of Amberlite IR-120 and eluted by citrate buffers of different pH: 3.25 and 4.25. The basic amino acids are being separated on a 15-cm column of the same resin but of different particle size, and eluted by 0.35 N citrate buffer pH 5.28. The influent buffer freed of air, is pumped at a constant rate through a column of sulfonated polystyrene resin. The effluent is met by a capilary stream of ninhydrin reagent delivered by a second pomp. The color is developed by passing the mixture of reagent and effluent through a spiral of cappilary tubing immersed in a boiling water bath. The absorbance of the resulting solution is measured continously at 570 and 440 mu. The peaks of the amino acids on the recorded curves can be

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integrated with great precision. The obtained curves showed with certainty the presence of the following amino-acids and related compounds: aspartic acid, threonine, srine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, ammonia and arginine. Cystoine was not found in the investigated materials. Among the amino acids detected, glutamic acid and proline showed the highest concentration. The obtained results will be discussed later because of the need of confirmation the reproducibility.

3. Determination of free amino acids:

The investigated material was also analyzed without any previously hydrolysis. A similar procedure was used as mentioned above. No free amino acids could be found except traces of leucine and phenylalanine.

4. Spectrophotometric estimations:

The visible and ultraviolet absorption spectra /200-700 mu/ of the protein-containing corrinoids were measured using an automatic Unicam SP-700 spectrophotometer. The obtained spectra differed from the characteristic spectrum of vitamin B_{12} as well as of that reported by Hedbom /2/ as being typical for a polypeptide containing corrinoids. No significant differences were found between the spectra of the two protein-complexes of corrinoids. Both however showed a disappearance of a small peak in the range of 280 mu as compared with the spectrum of cristalline vitamin B_{12}. The peaks usually recorded in the range of 360 and 550 mu were moved in the direction of ultraviolet hight.

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5. Ionophoretic studies according to Holdsworth /15/

Paper ionophoresis has been done with Whatman No 3 paper, using acidic solvent pH 2.7 consisting of 1 N CH_3COOH to which KCN was added. Separation was carried out over 10 hrs. with a voltage of 220 v /potencial gradient 9 volts per cm/. Each of the material being analyzed seperated into three components. The predominant electroneutral fraction moved into the position of cristalline vitamin B_{12}. From the remaining basic fractions one showed a yellowish-brown color whilest the other was pink. In order to get a clear-cut on the nature ~~of the nature~~ of the isolated protein containing corrinoids, further studies are required including identification of the structure of these compounds and their biological functions.

Plan of future work:

1. A multiple repetition of the experiments described in this report is required in order to get a quantitative and qualitative picture of the amino acid composition of the isolated protein containing corrinoids. All operations will be performed in the dark and the results compared with those obtained in the presence of light.

2. The biological activity /growth factor activity/ of the isolated compounds will be measured in microbiological tests and compared with that of different corrinoids.

3. The most suitable composition of the growth medium will be elaborated.

4. Attempts will be also made concerning the modification of the isolation and purification procedures being used. Other method will also be applied. Research will be carried

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out in order to isolate the holoenzymes /B_{12}-enzymes/.

5. Different fractions obtained after ionophoresis of the isolated material will be subjected to further studies.

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